

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
23 October 2003 (23.10.2003)

PCT

(10) International Publication Number  
WO 03/087772 A2

(51) International Patent Classification<sup>7</sup>: G01N

(21) International Application Number: PCT/US03/11253

(22) International Filing Date: 10 April 2003 (10.04.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/319,184 12 April 2002 (12.04.2002) US

(71) Applicant: COLORADO SCHOOL OF MINES  
[US/US]; 1500 Illinois Street, Golden, CO 80401 (US).

(72) Inventors: MADONNA, Angelo, J.; 6985 South Chase  
Court, Littleton, CO 80128 (US). VOORHEES, Kent, J.;  
714 Partridge Circle, Golden, CO 80401 (US). REES, Jon,  
C.; 1913 19th Street, #112, Golden, CO 80401 (US).

(74) Agent: KULISH, Christopher, J.; Holland & Hart LLP,  
P.O. Box 8749, Denver, CO 80201-8749 (US).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,  
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,  
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,  
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,  
MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD,  
SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ,  
VC, VN, YU, ZA, ZM, ZW.

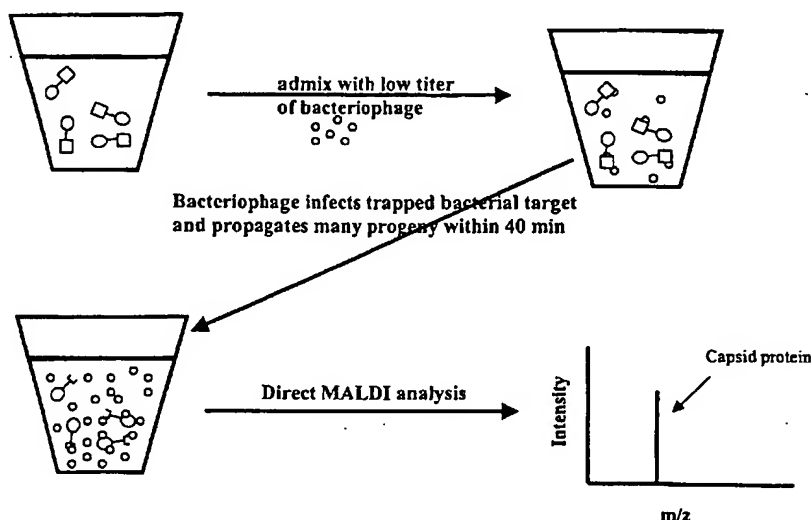
(84) Designated States (*regional*): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),  
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),  
European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE,  
ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, SE, SI,  
SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN,  
GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished  
upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.

(54) Title: METHOD FOR DETECTING LOW CONCENTRATIONS OF A TARGET BACTERIUM THAT USES PHAGES TO  
INFECT TARGET BACTERIAL CELLS



(57) Abstract: The invention is directed to a method for detecting low concentrations of bacteria in liquid solution that may or may not be complex liquid solutions. In one embodiment, immunomagnetic separation (IMS) is used to separate target bacterium that may be in a liquid mixture from other constituents in the mixture. A low concentration of a bacteriophage for the target bacteria is subsequently used to infect target bacterial cells that have been captured using the IMS technique. If at least a certain concentration of target bacterium are present, the bacteriophage will multiply to a point that is detectable. Matrix assisted laser desorption ionization/time-of-flight-mass spectrometry (MALDI/TOF-MS) is then used to produce a mass spectrum that is analyzed to determine if one or more proteins associated with the bacteriophage are present, thereby indirectly indicating that target bacterium were present in the liquid mixture.

WO 03/087772 A2

**METHOD FOR DETECTING LOW CONCENTRATIONS  
OF A TARGET BACTERIUM THAT USES PHAGES  
TO INFECT TARGET BACTERIAL CELLS**

5 **CROSS REFERENCE TO RELATED APPLICATIONS**

This application claims the benefit of United States Provisional Application No. 60/319,184, entitled "METHOD OF DETECTING LOW CONCENTRATIONS OF A TARGET BACTERIA THAT USES PHAGES TO INFECT TARGET BACTERIAL CELLS" and filed by Angelo J. Madonna and Kent J. Voorhees on April 12, 2002, which  
10 application is incorporated by reference into this application in its entirety.

**FIELD OF THE INVENTION**

The present invention relates to a method for detecting low concentrations of a target bacterium in a liquid mixture that uses bacteriophages to infect target bacterial cells.

15

**BACKGROUND OF THE INVENTION**

Standard microbiological methods have relied on substrate-based assays to test for the presence of specific organisms (Bordner, et al. 1978). These techniques offer very high levels of selectivity but are hindered by the requirement to first grow or cultivate pure  
20 cultures of the targeted organism, which can take 24 hours or longer. This time constraint severely limits the effectiveness to provide a rapid response to the presence of virulent strains of microorganisms.

Molecular biology techniques are quickly gaining acceptance as valuable alternatives to standard microbiological tests. In particular, serological methods have been widely  
25 employed to evaluate a host of matrices for targeted microorganisms (Kingsbury & Falkow 1985; Wyatt et al. 1992). These tests focus on using antibodies to first trap and then separate targeted organisms from other constituents in complicated biological mixtures. Once isolated, the captured organism can be concentrated and detected by a variety of different techniques that do not require cultivating the biological analyte.

30 One very popular approach, termed immunomagnetic separation (IMS), involves immobilizing antibodies to spherical, micro-sized paramagnetic beads and using the antibody-coated beads to trap targeted microorganisms from liquid media. The beads are easily manipulated under the influence of a magnetic field facilitating the retrieval and concentration of targeted organisms. Moreover, the small size and shape of the beads allow

them to become evenly dispersed in the sample, accelerating the rate of interaction between bead and target. These favorable characteristics lead to reductions in assay time and help streamline the analytical procedure making it more applicable for higher sample throughput and automation.

5 Downstream detection methods previously used with IMS include ELISA (Cudjoe, et al. 1995), dot blot assay (Skjerve et al. 1990), electrochemiluminescence (Yu and Bruno 1996), and flow cytometry (Pyle, et al. 1999). Although these tests provide satisfactory results, they are laborious to perform and give binary responses (yes/no) that are highly susceptible to false-positive results due to cross-reactivity with non-target analytes. Recently  
10 reported is a rapid method for identifying specific bacteria from complex biological mixtures using IMS coupled to matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS)(Madonna et al. 2001). This approach allowed a variety of matrices to be evaluated for the presence of a *Salmonella* species within a total analysis time of 1 hour. Moreover, the developed procedure required little sample processing, was  
15 relatively easy to perform, and the molecular weight information obtained made it possible to discriminate between signals from the target bacteria and signals from cross-reacted constituents.

MALDI-TOF-MS is a proven technique for identifying whole cellular microorganisms (Holland et al (1996); van Barr 2000; Madonna et al. 2000). In principle,  
20 MALDI is a 'fingerprinting' technique where mass spectra featuring varying distributions of protein signals are generated. The signature profiles that are produced, due to inherent differences in microbial proteomes, make it possible to discriminate between organisms down to the strain level (Arnold and Reilly 1998). The MALDI-TOF technique coupled with IMS includes, in one embodiment, mixing immunomagnetic beads specific to the target bacteria  
25 with the liquid mixture that may contain the target bacteria for a short incubation period (e.g., 20 min). Any target bacteria captured by the beads are washed twice, re-suspended in deionized H<sub>2</sub>O, and directly applied onto a MALDI sample probe. The target bacteria-bead complex is then overlaid with a micro-volume of matrix solution and dried at room temperature. Irradiation of the resulting crystalline mass with a high intensity laser promotes  
30 the liberation and ionization of intact cellular proteins that are subsequently detected by a TOF mass spectrometer. The resulting mass spectrum is then interrogated for definitive mass peaks that signify the presence of the target bacteria.

## SUMMARY OF THE INVENTION

The invention is directed to a method for determining if a target bacterium is present in a liquid solution when the target bacterium is or may be present in a low concentration that is at or near the detection limit for a particular detection technology. As used herein, the term "target bacterium" refers to a specie of species of bacteria. In turn, the invention is applicable to situations in which it is desirable to determine whether a target bacterium (e.g., *E. coli*) is present in a liquid solution when the number of target bacterium per unit volume of solution (i.e., the concentration of the target bacterium) is or may be below the detection limit for a particular detection technology. In some instances, a plurality of target bacterium may be referred to as the target bacteria.

In one embodiment, the process comprises using a biological amplification procedure in which bacteriophages for the target bacterium are applied to the liquid solution. (Bacteriophages are viruses that infect bacteria and in the process produce many progeny. Structurally, the bacteriophage consists of a protein shell (capsid) that encapsulates the viral nucleic acid. The capsid is constructed from repeating copies of the same protein. Bacteriophages are able to infect specific bacterial cells and because of the multiplication of the genetic material, the cells eventually burst releasing millions of copies of the original phage.) The bacteriophages and any of the target bacterium present in the liquid solution are allowed to incubate. During the incubation period, the bacteriophages will multiply by infecting target bacterium present in the solution. More specifically, the bacteriophage replicates numerous copies of itself in an infected target bacterium. Eventually, the infected target bacterium lyses and the replicated or progeny bacteriophages are released into the liquid solution. The solution is then analyzed to determine if a biomarker for the bacteriophage is present, thereby indirectly indicating that the target bacterium is present in the liquid solution. Possible analysis techniques comprise mass spectrometry techniques, such as MALDI-MS and electro-spray ionization-MS techniques.

To assure that the detection of a biomarker for the bacteriophage indicates that the target bacterium is present in the liquid solution, a concentration of the bacteriophage is applied to the liquid solution that is below the detection limit for the biomarker for the bacteriophage for whatever analysis technique is employed. This assures that if the biomarker for the bacteriophage is detected by the analysis technique, the detectable concentration of the biomarker is attributable to the replication of the bacteriophage by the

target bacterium present in the liquid solution. In certain situations, the use of such a concentration of bacteriophage has a multiplicity of infection ("MOI") (i.e., ratio of infecting bacteriophages to target bacterium) that is too low to rapidly produce a sufficient concentration of bacteriophages or biomarkers for the bacteriophage for detection.

5        Another embodiment of the process addresses this problem by adding a very high concentration of the bacteriophage to the liquid solution, thereby assuring a high MOI. In this case, the concentration of the bacteriophage added to the solution may exceed the detection limit of whatever analysis technique is employed to detect the bacteriophage or biomarker of the bacteriophage. Consequently, the process applies parent bacteriophage to  
10       the solution that can distinguished from any progeny bacteriophage resulting from the infection of target bacterium in the mixture. If the distinguishable progeny bacteriophage or a distinguishable biomarker of the progeny bacteriophage are present, this indicates that the target bacterium is present in the solution.probe.

      In one embodiment, the parent bacteriophage (i.e., the bacteriophage initially applied  
15       to the solution) are "tagged" so that whatever analysis technique is employed is inherently capable of distinguishing the parent bacteriophage or parent bacteriophage biomarkers from the progeny bacteriophage or biomarkers for the progeny bacteriophage. For example, if a mass spectral analysis technique is employed, the parent bacteriophage are "tagged" with a substance that alters or shifts the mass spectrum of the parent bacteriophage relative to the  
20       progeny bacteriophage, which will not inherit the "tag" from the parent bacteriophage. For example, a biotinylated bacteriophage can be employed as a parent bacteriophage and will have a different mass spectrum than the progeny bacteriophage produced by the biotinylated bacteriophage infecting target bacterium present in the solution. Other "tags" can be employed for other types of analytical techniques.

25       In another embodiment, the parent bacteriophage possesses a characteristic that allows the parent bacteriophage to be separated from the progeny bacteriophage in the liquid solution prior to analysis, thereby assuring that most, if not all of the bacteriophages present in the liquid solution after separation are progeny bacteriophage resulting from the replication of the parent bacteriophage by target bacteria present in the liquid solution. In one  
30       embodiment, the parent bacteriophages initially applied to the liquid solution are biotinylated bacteriophages. Biotinylated bacteriophages are highly attracted to strepavidin. This attraction is exploited to separate the biotinylated bacteriophage from progeny bacteriophage

resulting from replication of the biotinylated bacteriophage by target bacterium present in the mixture.

In one embodiment, the biotinylated bacteriophage are attached to a streptavidin coated probe. Consequently, separation of the biotinylated bacteriophage from the liquid solution after the incubation period is accomplished by removing the probe from the liquid solution. In another embodiment, streptavidin-coated magnetic beads are applied to the liquid solution. The beads are used to pick up the biotinylated bacteriophage. The beads are then separated from the liquid solution using a magnet. In yet another, embodiment a streptavidin coated probe (e.g., a slide) is applied to the liquid solution after the incubation period. The biotinylated bacteriophage adhere to the probe and then the probe is separated from the liquid solution.

Yet a further embodiment of the invention recognizes that the liquid solution in which the target bacterium may be present is or may be a complex mixture that includes biological material that makes the detection of the bacteriophage or biomarker for the bacteriophage more difficult or reduces the reliability of the information provided by the detection technology employed. For instance, when a mass spectrometry detection methodology is employed, the complex mixture may produce a signal in which the biomarker associated with the bacteriophage is obscured or, stated differently, has a low signal-to-noise ratio. To address this possibility, the liquid solution is subject to a purification step in which target bacterium that are present in the liquid solution are separated from the remainder of the solution. In one embodiment, immuno-magnetic separation ("IMS") is utilized to separate target bacterium present in the liquid solution from the remainder of the solution. In one particular embodiment, magnetic beads are coated with an antibody for the target bacterium. The antibodies pick up the target bacterium present in the liquid mixture and then a magnet is used to separate the beads from the remainder of the liquid solution. The beads and any adhering target bacterium are then subjected to the biological amplification process and analysis. It should be appreciated this purification step also addresses the possibility that feral versions of the bacteriophage may be present in the liquid solution and that such versions could produce a false positive if the liquid solution was not subjected to a purification step.

If feral versions of the bacteriophage are not of concern, the purification step can be implemented after the biological amplification process. In this embodiment, the purification

step involves separating the bacteriophages and the liquid solution, rather than separating the target bacterium and the liquid solution. In one embodiment, an IMS is used in which magnetic beads are coated with an antibody for the bacteriophage. The beads pick up the bacteriophages present in the solution and then a magnet is used to separate the beads from the remainder of the solution.

In another embodiment of the invention, the analysis step comprises using MS/MS analysis to determine if a biomarker for the target bacterium is present. The use of MS/MS analysis produces a highly reliable indication of the presence of a biomarker for a target bacterium. As a consequence, at least in some cases, the use of MS/MS analysis renders the need for a purification step unnecessary.

In yet another embodiment, the invention is directed to a process for detecting low concentrations of a target bacterium in complex mixtures. In one embodiment, the process comprises using an IMS procedure to isolate at least some of a target bacterium that may be present in a liquid mixture. The process further includes employing a biological amplification procedure in which a low titer or concentration of bacteriophages for the target bacterium are applied to at least some of the target bacterium that has been isolated by the IMS procedure. The mixture of bacteriophages and any of the target bacterium that has been isolated is allowed to incubate. If at least a certain concentration of the target bacterium is present, the bacteriophages will multiply during the incubation period such that a high titer or concentration of bacteriophages will be present in the mixture and detectable by MALDI-TOF-MS analysis. If no or only a small number of the target bacterium is present, there will be a low concentration of bacteriophages present in the mixture that will not be reasonably detectable by MALDI-TOF-MS analysis. Following incubation, a MALDI-TOF-MS analysis is performed on the incubated mixture of bacteriophages and target bacterium. The resulting mass spectrum is analyzed to determine if a protein that is associated with the bacteriophages is present. If the protein for the bacteriophage is detected, then it can be concluded that at least a low concentration of the target bacterium is present in the mixture.

It should also be appreciated that the method of the invention is capable of detecting low concentration of a target bacterium regardless of the manner in which the bacterium was grown or propagated.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts, for one embodiment of the invention, an immunomagnetic bead that is used to isolate the target microorganism (antibodies not drawn to scale);

Figure 2 is a schematic representation of the immunomagnetic purification step used to isolate a target antigen (*Escherichia coli*) in one embodiment of the invention;

Figure 3 is a schematic representation of the bacteriophage amplification step for one embodiment of the invention;

Figure 4 shows a typical MALDI-TOF mass spectrum obtained from a high titer sample of MS2 in PBS; and

Figures 5A-5C illustrate how an embodiment of the invention was able detect a biomarker of the bacteriophage indicative of presence of *E. coli* for decreasing concentrations of *E. coli*.

## DETAILED DESCRIPTION

Generally, the invention relates to the use of a bacteriophage to indirectly detect the presence of a target bacterium in a liquid solution where the concentration of the target bacterium is or is likely to be near or below the detection limit for the particular detection technology employed.

Bacteriophages are viruses that infect bacteria and in the process of infecting the bacteria produce many progeny. Structurally, the bacteriophage consists of a protein shell (capsid) that encapsulates the viral nucleic acid. The capsid is constructed from repeating copies of the same protein(s). Bacteriophages are able to infect specific bacterial cells and because of the multiplication of the number of progeny, the cells eventually burst releasing millions of copies of the original phage. This infection process has been utilized to serve as a biomarker amplification step for detecting low concentrations of target bacterial cells. For example, the capsid of the MS2 bacteriophage contains 180 copies of a 13 kDa protein. This particular virus specifically infects strains of *Escherichia coli* and is able to produce between 10,000 to 20,000 copies of itself within 40 min after attachment to the target bacterial cell. Essentially, one *E. coli* could be infected with MS2 resulting in the replication of the capsid protein(s) by a factor of  $1.8 \times 10^6$ .

The results from matrix assisted laser desorption ionization/mass spectrometry (MALDI/TOF) can be used to show the utility of the amplification step. MALDI-TOF-MS is



a proven technique for identifying whole cellular microorganisms (Holland et al 1996; van Barr 2000; Madonna et al. 2000). In principle, MALDI is a 'fingerprinting' technique where mass spectra featuring varying distributions of protein signals are generated. The signature profiles are produced due to inherent differences in microbial proteomes that make it possible  
5 to discriminate between organisms down to the strain level (Arnold and Reilly 1998).

In an experiment where the protein MALDI signal from target bacterial cells was too weak for detection, the addition of low levels (too low to detect by MALDI) of the appropriate phage to the target bacterial cells after about thirty minutes produced a detectable protein MALDI signal attributable to the phage capsid protein. Bacteriophages specific for  
10 other bacterial species typically have capsid proteins of different molecular weight and therefore give a different MALDI signal. Therefore, the procedure is applicable to a multitude of different bacterial species. Other detection technologies, such as ion mobility spectrometry, optical spectroscopy, immuno techniques, chromatographic techniques and aptamer processes, are also feasible.

Generally, the process for detecting low concentrations of a target bacterium in a complex liquid mixture that contains or is likely to contain biological material other than the target bacterium comprises processing the mixture or a portion thereof to produce a liquid mixture, solution or sample for analysis that, if at least a certain concentration of the target bacterium is present in the mixture, a discernable signal or indication thereof is produced. It  
15 should be understood that the terms "liquid solution" and "liquid mixture" refer to the original solution or mixture that is the subject of the test and any liquid solutions or mixtures that, as a result of the application of the method, contain a portion of the original solution or mixture.

In one embodiment, the process comprises making a determination if a low concentration of a target bacterium is present in the liquid solution. This determination can  
25 be made by assuming that any of the target bacterium that are present in the liquid solution are present in a low concentration. Alternatively, an assay can be performed to determine if the target bacterium is present in a concentration that reliably exceeds the detection limit of whatever detection technology is being utilized. For instance, a mass spectrometry technique can be utilized. If the mass spectrometry technique provides a reliable signal indicative of the  
30 presence of the target bacterium in the liquid solution, no further steps needs to be taken. If, however, the mass spectrometry technique does not provide a reliable signal indicative of the presence of the target bacterium in the liquid solution, then it can be concluded that the target

bacterium may be present in the liquid solution but in a concentration that below or near the detection limit of the mass spectrometry technique. In this case, further steps are taken to determine whether the target bacterium is present in the liquid solution in a concentration that is under the detection limit of the mass spectrometry technique.

5           The process involves a purification step that involves capturing the target bacterium that may be present in the mixture and separating any of the captured bacterium from other biological material that may be present in the mixture. By separating any of the captured target bacterium from other biological material that may be present in the mixture, the portion of the subsequently produced mass spectrum signal associated with other biological material  
10 present in the mixture is reduced. In one embodiment, an immunomagnetic separation (IMS) technique is used to capture and separate the target bacterium.

          The process further comprises subjecting at least some of any of the captured and separated target bacterium to an amplification step in which the target bacterium are infected with a bacteriophage that is specific to the target bacterium. If there is at least a certain  
15 concentration of the target bacterium present, the bacteriophage will multiply to a point that a biomarker associated with the bacteriophage will be detectable using an analysis technique, such as MALDI/TOF-MS. In the case of MALDI/TOF-MS, a portion of the subsequently produced mass spectral signal indicative of the presence of the bacteriophage will be increased. If there is less than a particular concentration of the target bacterium present, the  
20 bacteriophage will not multiply sufficiently to be detectable in the mass spectrum produced using MALDI/TOF-MS. In essence, provided there is at least a certain concentration of the target bacterium present in the mixture, the purification and amplification steps serve to increase the signal-to-noise ratio for the portion of the subsequently produced mass spectrum that is associated with the bacteriophage.

25           After amplification, at least a portion of the amplified mixture is subjected to analysis to determine if a biomarker for the target bacterium is present. For example, MALDI/TOF-MS analysis can be used to produce a mass spectrum. The mass spectrum is analyzed to determine if one or more biomarkers for the bacteriophage are present. If such biomarkers are present, this is an indirect indication that at least a certain concentration of the target  
30 bacterium was present in the originally sampled mixture.

          It should be appreciated that the need for the purification step may not be necessary in situations in which the portion of the signal associated with other biological materials can be

filtered, eliminated or otherwise ignored and/or in situations in which the amplification step has a gain such that the portion of the mass spectrum associated with the bacteriophage is likely to exceed any background signal associated with other biological materials and/or situations in which a false positive is considered to be remote. Further, it should also be appreciated that one of the other biological materials that can make it difficult to determine if the biomarker for the bacteriophage is present is a wild version of the bacteriophage that is present in the liquid solution that is being tested. The purification step addresses the presence of a wild bacteriophage.

If the presence or possible presence of a wild bacteriophage in the solution or mixture being tested is not a concern, it is also possible to perform a purification step after the amplification step. However, in this case, the bacteriophage is separated from the remainder of the liquid solution. The previously noted IMS technique can be employed. However, the magnetic bead is coated with an antibody for the bacteriophage, rather than an antibody for the target bacterium.

It is also possible in certain cases to eliminate the need for a purification step by utilizing a highly specific analysis technique, such as MS/MS, in which the biomarker for the bacteriophage is so highly specific to the bacteriophage that there is little likelihood of a false positive. Currently, the use of MS/MS is limited to capsid proteins that are less than 7000 Da.

With reference to Fig. 1, the immunomagnetic separation (IMS) technique for capturing the target bacterium in a mixture and separating any captured target bacterium from other biological material in the mixture is described. Microsize beads are constructed from an iron oxide core coated with a polymeric surface. Secondary antibodies raised against the Fc region of the primary antibodies are covalently attached via a linker to the polymer surface. The primary antibody (raised against a targeted microorganism) is attached to the beads by strong noncovalent interactions with the secondary antibody, which holds the primary antibody in the proper orientation for reaction with the targeted antigen.

With reference to Fig. 2, the immunomagnetic beads are added to the bacterial or biological mixture that is the subject of the analysis and incubated for 20 minutes at room temperature. The beads are then isolated to the side of the reaction tube using a magnet. This process allows the extraneous (non-targeted) material to be removed by aspiration. At this stage, the beads can be washed several times prior to re-suspending them in PBS.

With reference to Fig. 3, the bead-bacterial target complex is admixed with a low titer suspension of bacteriophage specific to the targeted bacterium. The titer is held low so that the mass spectrometry signal from the virus is non-detectable. After a 40-minute incubation, the bacteriophage have completed a propagation cycle of attachment, insertion, self-assembly, and cell lysis resulting in the production of many progeny that are released into the reaction milieu. The milieu is then analyzed to determine if a biomarker for the bacteriophage is present that indirectly indicates that the target bacterium was present. For instance, the milieu can be analyzed by MALDI-TOF-MS using a sandwich sample preparation technique with a ferulic acid matrix. Other MALDI matrices known in the art are also feasible. The resulting mass spectrum shows the presence of the bacteriophage capsid protein, which would not have been present if the target bacterium was not also present.

An IMS technique for capturing target bacterium in a mixture and separating any captured target bacterium from other biological material in the mixture and subsequent MALDI-TOF/MS analysis are described in U.S. patent application serial no. 10/063,346, entitled "Method for Determining if a Type of Bacteria is Present in a Mixture," filed on April 12, 2002, which is incorporated herein, in its entirety, by reference.

### Example

As described hereinafter, an embodiment of the method has been used to reduce the detection limit for *E. coli* to less than  $5.0 \times 10^4$  cells  $\text{mL}^{-1}$ . The method used immunomagnetic beads coated with antibodies against *E. coli*, hereinafter referred to as the target-bead complex, to isolate the bacterium from solution. The target-bead complex was then re-suspended in a solution containing MS2, a bacteriophage that is specific for *E. coli*. The MS2 bacteriophage concentration was adjusted so that the ion signal from the capsid protein of the MS2 bacteriophage was below the detection limit of the mass spectrometer. After a 40-minute incubation period, an aliquot of the solution was removed and analyzed by the on-probe MALDI-TOF-MS procedure for the 13 kDa capsid protein. The  $[\text{M}+\text{H}]^+$  ( $m/z$  13,726) and  $[\text{M}+2\text{H}]^{+2}$  ( $m/z$  6865) ion signals for the MS2 capsid protein were detected (Figure 4).

With reference to Fig. 5A, application of the process to a mixture that contains a concentration of  $5.0 \times 10^6$  *E. coli* cells  $\text{mL}^{-1}$  yields a mass spectrum with protein signals for

both *E. coli* and the MS2 bacteriophage. The process was repeated for decreasing concentrations of *E. coli*. Specifically, with reference to Fig. 5B, the process was repeated for a concentration of  $\sim 5.0 \times 10^5$  *E. coli* cells mL<sup>-1</sup>. In this case, the mass spectrum fails to definitively show any protein signals for the *E. coli* cells, but does show the protein signals for the MS2 bacteriophage capsid protein. With reference to Fig. 5C, the process was repeated for an *E. coli* concentration of  $\sim 5.0 \times 10^4$  cells mL<sup>-1</sup>. In this case, the mass spectrum fails to definitively show any protein signals for the *E. coli* cells but still show protein signals for the MS2 bacteriophage capsid protein. These results indicate that *E. coli* was trapped by the immunomagnetic beads and then infected by the MS2 virus, which was able to multiply and increase the concentration of the capsid protein to a detectable level. Presently, target bacterium concentrations of as low as  $\sim 1.0 \times 10^3$  cells mL<sup>-1</sup> have been indirectly detected using this process.

The following describes various aspects of the embodiment of the method implemented with respect to the example of the detection of *E. coli*.

#### ***E. coli* Preparation**

The *E. coli* bacteria were grown in trypticase soy broth (TSB) (Difco, Detroit, MI) with incubation at 37° C using standard microbial methods.

#### **Bacteriophage Propagation**

Bacteriophage propagation was performed in accordance to the Adams agar-overlay method as described in M. H. Adams' Bacteriophages (Interscience Publishers, Inc., New York, 1959). Briefly, a soft-agar/host covering was prepared by overlaying agar plates (trypticase soy agar, Difco) with a 2.5 mL of melted 0.5% agar (same medium), which contained two drops of a 20 hr host in TSB. The soft-agar covering was allowed to harden before the addition of a 0.5 mL overlay of a concentrated suspension of MS2, prepared by rehydrating freeze-dried MS2 in TSB. After 24 hours, the soft agar was scraped off the surface of the agar plates and centrifuged (1000G) for 25 min to sediment the cellular debris and agar. The supernatant was conserved, passed through 0.22 µm Millipore filters, and stored by refrigeration at 4-8° C.

### Immunomagnetic Bead Preparation

Rabbit anti-E. coli IgG antibodies (Cortex Biochem, San Leandro, CA) were attached to the immunomagnetic beads (MagaBeads, Goat anti-Rabbit IgG F(c), Cortex Biochem) using the manufacturer's suggested protocol.

5

### Immunomagnetic separation (IMS) E. coli

Escherichia coli were isolated from aqueous suspensions by affinity capture using the immunomagnetic beads. Suspensions of bacteria were prepared in 1.5mL microcentrifuge tubes (Brinkmann Instruments, Inc., Westbury, Ny) by combining 100  $\mu$ L of broth media with 900  $\mu$ L of phosphate buffer saline (PBS, 0.01M  $\text{Na}_2\text{HPO}_4$ , 0.15M NaCl titrated to pH 7.35 with HCl). Cell concentrations were determined using a Petroff-Hauser counting chamber (Hauser Scientific, Horsham, PA).

The immunomagnetic separation (IMS) procedure developed in this investigation involved the following steps: In the first step, a 30  $\mu$ L aliquot of the immunomagnetic beads were added to the bacterial sample solution and incubated for 20 minutes at room temperature with continuous shaking. The second step involved concentrating the beads to the side of the sample tube using a magnetic particle concentrator (DynaL, Lake Success, NY) and removing the supernatant using a 1mL pipette. In the third step, the magnet was removed and the beads were re-suspended in 1mL of fresh PBS with vigorous shaking for 20 sec to wash away any nonspecifically adhering components. The bead suspension was then transferred to a new tube and steps 2 and 3 repeated one more time. In the fourth and final step, the beads were isolated with the magnet followed by decanting the buffer wash to waste and re-suspending the beads in 500  $\mu$ L of deionized water. Subsequently, the bead-E. coli complexes were admixed with a low titer (below the detection limit of the mass spectrometer) of the MS2 bacteriophage and incubated at room temperature with gentle shaking for 40 minutes. An aliquot of the suspension was then removed and analyzed for the MS2 capsid protein using a sandwich sample preparation with a ferulic acid matrix (12.5mg of ferulic acid in 1mL of 17% formic acid: 33% acetonitrile: 50% deionized  $\text{H}_2\text{O}$ ).

30

### MALDI-TOF MS

All mass spectra were generated on a Voyager-DE STR+ (AB Applied Biosystems, Framingham, MA) MALDI-TOF mass spectrometer, operating in the positive linear mode.

The following parameters were used: accelerating voltage 25 kV, grid voltage 92% of accelerating voltage, extraction delay time of 350 nsec, and low mass ion gate set to 4 kDa. The laser intensity ( $N_2$ , 337 nm) was set just above the ion generation threshold and pulsed every 300ns. Mass spectra were acquired from each sample by accumulating 100 laser shots  
5 from five different sample spots (final spectrum = average of 5.x 100 laser shots).

It should be appreciated that to assure that the detection of a biomarker for the bacteriophage indicates that the target bacterium is present in the liquid solution, a concentration of "parent" bacteriophage is applied to the liquid solution that is below the  
10 detection limit for the bacteriophage or biomarker for the bacteriophage for whatever analysis technique is employed. This assures that if the bacteriophage or the biomarker for the bacteriophage is detected by the analysis technique, the detectable concentration of the bacteriophage or biomarker is attributable to progeny bacteriophage, i.e., bacteriophage resulting from the replication of the bacteriophage by the target bacterium present in the  
15 liquid solution. In certain situations, the use of such a concentration of "parent" bacteriophage has a multiplicity of infection ("MOI") (i.e., the ratio of the number of parent bacteriophage to the number of target bacterium) that is too low to produce a sufficient concentration of bacteriophages or biomarkers for the bacteriophage for detection.

To overcome the drawbacks associated with a low MOI, a sufficiently high  
20 concentration of "parent" bacteriophage is added to the liquid solution. In this case, the concentration of the "parent" bacteriophage added to the solution may exceed the detection limit of whatever analysis technique is employed to detect the bacteriophage or biomarker of the bacteriophage. Consequently, analysis of a liquid solution treated in this manner could detect the "parent" bacteriophages that were added to the solution, rather than the progeny  
25 bacteriophage resulting from replication by the target bacteria.

Consequently, another embodiment of the process applies a concentration of "parent" bacteriophage to the solution that is capable of being distinguished from any progeny bacteriophage. In one embodiment, the parent bacteriophage (i.e., the bacteriophage initially applied to the solution) are "tagged" so that whatever analysis technique is employed is  
30 inherently capable of distinguishing the parent bacteriophage or parent bacteriophage biomarkers from the progeny bacteriophage or biomarkers for the progeny bacteriophage. For example, if a mass spectral analysis technique is employed, the parent bacteriophage are

"tagged" with a substance that alters or shifts the mass spectrum of the parent bacteriophage relative to the progeny bacteriophage, which will not inherit the "tag" from the parent bacteriophage. For example, a biotinylated bacteriophage is employed as a parent bacteriophage and has a different mass spectrum than the progeny bacteriophage produced by the biotinylated bacteriophage infecting target bacterium present in the solution. Other "tags" can be employed for other types of analytical techniques.

In another embodiment, the parent bacteriophage possesses a characteristic that allows the parent bacteriophage to be separated from any of the progeny bacteriophage in the liquid solution prior to analysis, thereby assuring that most, if not all of the bacteriophage present in the liquid solution after separation are progeny bacteriophage resulting from the replication of the parent bacteriophage by target bacteria present in the liquid solution. In one embodiment, biotinylated bacteriophage are initially applied to the liquid solution. Biotinylated bacteriophage are highly attracted to strepavidin. Consequently, to separate the biotinylated bacteriophage from the liquid solution a strepavidin probe is utilized. In one embodiment, the biotinylated bacteriophage are attached to a strepavidin coated probe and the probe is placed in the liquid solution. In this case, separation of the biotinylated bacteriophage from the liquid solution after the incubation period is accomplished by removing the probe from the liquid solution. In another embodiment, strepavidin-coated magnetic beads are applied to the liquid solution. The biotinylated bacteriophages are attached to the strepavidin-coated magnetic beads prior to the application of the beads to the solution. Alternatively, the beads are used to pick up biotinylated bacteriophages that were previously added to the solution and then separated from the liquid solution using a magnet. In yet another, embodiment a strepavidin coated probe (e.g., a slide) is applied to the liquid solution after the incubation period. The biotinylated bacteriophages adhere to the probe and then the probe is separated from the liquid solution. Regardless of the manner in which the biotinylated bacteriophages are separated from the liquid mixture, at least a portion of the solution is then subjected to analysis to determine if the bacteriophage or a biomarker for the bacteriophage is present, which indirectly indicates that the target bacteria was present in the solution.



What is claimed is:

1. A method for determining if a target bacterium is present in a liquid solution when the target bacterium is or may be present in a low concentration that is near or below the detection limit for a particular detection technology comprising:

making a determination as to whether a low concentration of a target bacterium may be present in a liquid solution;

using, following said step of making and if a determination is made that a low concentration of said target bacterium may be present, a first quantity of a bacteriophage to infect at least some of any target bacterium that are present in said liquid solution and multiply the number of bacteriophage in said liquid solution; and

analyzing, following said step of using, at least a portion of said liquid solution to determine if a biomarker for said bacteriophage is present that indirectly indicates that said target bacterium is also present in said liquid solution.

15

2. A method, as claimed in claim 1, wherein:

said step of making comprises assuming that any of said target bacterium that are present in said liquid solution are present in said liquid solution in a low concentration.

20 3. A method, as claimed in claim 1, wherein:

said step of making comprises performing an assay to determine if a biomarker for said target bacterium is present that is indicative of the presence of a concentration of said target bacterium that reliably exceeds the detection limit of the detection technology.

25 4. A method, as claimed in claim 3, wherein:

said step of performing an assay comprises performing a mass spectrum analysis.

5. A method, as claimed in claim 1, wherein:

said step of using comprises applying a first quantity of said bacteriophage to said liquid solution such that there is likely to be a high multiplicity of infection ("MOI") number.

30

6. A method, as claimed in claim 1, wherein:

said step of analyzing comprises performing a mass spectrum analysis.

7. A method, as claimed in claim 1, wherein:

said step of analyzing comprises performing a MALDI analysis.

5

8. A method, as claimed in claim 1, wherein:

said step of analyzing comprises performing a MALDI-TOF analysis.

9. A method, as claimed in claim 1, wherein:

10 said step of analyzing comprises performing an electro-spray ionization mass spectrometry analysis.

10. A method, as claimed in claim 1, wherein:

said step of analyzing comprises performing an ion mobility spectrometry analysis.

15

11. A method, as claimed in claim 1, wherein:

said step of analyzing comprises performing an optical spectroscopy analysis.

12. A method, as claimed in claim 1, wherein:

20 said step of analyzing comprises performing an immuno analysis.

13. A method, as claimed in claim 1, wherein:

said step of analyzing comprises performing a chromatographic analysis.

14. A method, as claimed in claim 1, wherein:

25 said step of analyzing comprises performing an aptamer analysis.

15. A method, as claimed in claim 1, further comprising:

30 determining whether said liquid solution is likely to contain a biological element with a biomarker that could create a false positive for a biomarker for said bacteriophage.

16. A method, as claimed in claim 15, wherein:

said step of determining comprises assuming that a biological element is present in said liquid solution that has a biomarker that could create a false positive for a biomarker for said bacteriophage.

5           17.    A method, as claimed in claim 15, wherein:

said step of determining comprises performing an assay to determine if a biomarker is present that could create a false positive for a biomarker for said bacteriophage.

10           18.    A method, as claimed in claim 15, further comprising:

after a determination has been made that said biological element may present, separating said target bacterium from said liquid solution.

            19.    A method, as claimed in claim 18, wherein:

15           said step of separating comprises using immuno-magnet beads coated with antibodies for said target bacteria.

            20.    A method, as claimed in claim 18, further comprising:

20           after a determination has been made said step of separating is performed prior to said step of using.

            21.    A method, as claimed in claim 18, wherein:

after a determination has been made that said biological element may present and after said step of using, separating said bacteriophage from said liquid solution.

25           22.    A method for determining if a target bacterium is present in a liquid solution when the target bacterium is or may be present in a low concentration that is near or below the detection limit for a particular detection technology comprising:

making a determination as to whether a low concentration of a target bacterium may be present in a liquid solution;

30           using, following said step of making and if a determination is made that a low concentration of said target bacterium may be present, a first quantity of a biotinylated

bacteriophage to infect at least some of any target bacterium that are present in said liquid solution and multiply the number of bacteriophage in said liquid solution; and

analyzing, following said step of using, said liquid solution to determine if a biomarker for said bacteriophage is present that indirectly indicates that said target bacterium is also present in said liquid solution.

23. A method, as claimed in claim 22, wherein:

said step of using comprises applying a first quantity of said biotinylated bacteriophage to said liquid solution such that there is likely to be a high multiplicity of infection ("MOI") number.

24. A method, as claimed in claim 22, wherein:

said step of using comprises applying a first quantity of said biotinylated bacteriophage to said liquid solution that is near or likely to exceed the multiplicity of infection ("MOI") number, wherein said first quantity of biotinylated bacteriophage are attached to a strepavidin coated probe.

25. A method, as claimed in claim 22, wherein:

said step of analyzing comprises separating a substantial portion of said biotinylated bacteriophage from said liquid solution to produce a remaining liquid solution.

26. A method, as claimed in claim 25, wherein:

said step of separating comprises using a strepavidin coated probe.

27. A method, as claimed in claim 26, wherein:

said step of using a strepavidin coated probe comprises separating a strepavidin coated probe from said liquid solution, said strepavidin coated probe having been used to apply said biotinylated bacteriophage to said liquid solution in said step of using.

28. A method, as claimed in claim 26, wherein:

said step of using a strepavidin coated probe comprises applying a strepavidin coated probe to said liquid solution to capture biotinylated bacteriophage in said liquid solution.

29. A method, as claimed in claim 25, wherein:  
said step of analyzing comprises assaying said remaining liquid solution to determine  
if a biomarker for said bacteriophage is present.

5

30. A method, as claimed in claim 29, wherein:  
said step of assaying comprises performing a mass spectrum analysis.

10

31. A method, as claimed in claim 22, further comprises:  
separating said target bacterium from said mixture.

32. A method, as claimed in claim 31, wherein:  
said step of separating is performed prior to said step of using.

15

33. A method, as claimed in claim 22, further comprises:  
separating said biotinylated bacteriophage from said mixture.

34. A method, as claimed in claim 33, wherein:  
said step of separating is performed after said step of using.

20

35. A method for determining if a target bacterium is present in a liquid solution  
when the target bacterium is or may be present in a low concentration that is near or below  
the detection limit for a particular detection technology comprising:

first determining whether a low concentration of a target bacterium may be present in  
a liquid solution;

25

second determining whether a biological element may be present in said liquid  
solution that has a biomarker that could create a false positive for a biomarker for a  
bacteriophage used in said subsequent step of using and used in said step of analyzing as an  
indirect biomarker for the presence of said target bacterium;

30

first using, following said step of first determining and when a determination has been  
made that a low concentration of said target bacterium may be present, a first quantity of a

biotinylated bacteriophage to infect at least some of any target bacterium that are present in said liquid solution and multiply the number of bacteriophage in said liquid solution;

second using, following said step of second determining and when a determination has been made that a biological element may be present, purification technique; and

5 analyzing, following said steps of first using and second using, said liquid solution to determine if a biomarker for said bacteriophage is present that indirectly indicates that said target bacterium is also present in said liquid solution.

36. A method, as claimed in claim 35, wherein:

10 said step of second using comprises performing a purification technique that separates at least some of any target bacterium that are present in said liquid solution from said liquid solution.

37. A method, as claimed in claim 35, wherein:

15 said step of performing occurs before said step of first using.

38. A method, as claimed in claim 37, wherein:

said step of performing comprises using immuno-magnetic beads to which are attached antibodies for said target bacterium.

20

39. A method, as claimed in claim 35, wherein:

said step of second using comprises performing a purification technique that separates said bacteriophage from said liquid mixture.

40. A method, as claimed in claim 39, wherein:

25 said step of performing occurs after said step of first using.

41. A method, as claimed in claim 39, wherein:

30 said step of performing comprises using immuno-magnetic beads to which are attached antibodies for said bacteriophage.

42. A method for determining if a target bacterium is present in a liquid solution when the target bacterium is or may be present in a low concentration that is near or below the detection limit for a particular detection technology comprising:

making a determination as to whether a low concentration of a target bacterium may  
5 be present in a liquid solution;

using, following said step of making and if a determination is made that a low concentration of said target bacterium may be present, a first quantity of a tagged bacteriophage to infect at least some of any target bacterium that are present in said liquid solution and produce a number of untagged bacteriophage in said liquid solution; and

10 analyzing said liquid solution to determine if a biomarker for said untagged bacteriophage and that is distinguishable relative to any biomarkers for said tagged bacteriophage is present and that indirectly indicates that said target bacterium is also present in said liquid solution.

15 43. A method, as claimed in claim 42, wherein:

said step of analyzing comprises separating said tagged bacteriophage from said liquid solution..

44. A method, as claimed in claim 42, wherein:

20 said step of analyzing comprises using a mass spectrometry analysis technique to produce a mass spectrum.

45. A method, as claimed in claim 44, wherein:

25 said step of using comprises inspecting said mass spectrum to determine if a biomarker that is unique to said untagged bacteriophage relative to said tagged bacteriophage is present.

FIGURE 1

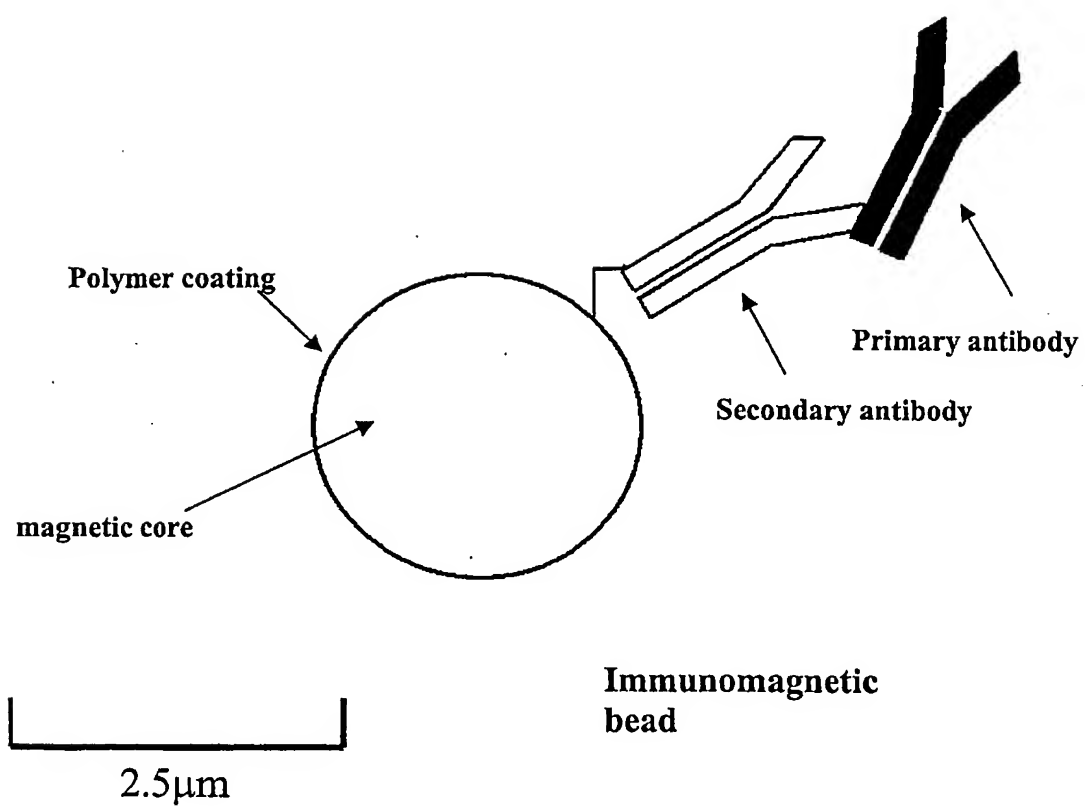




FIGURE 2

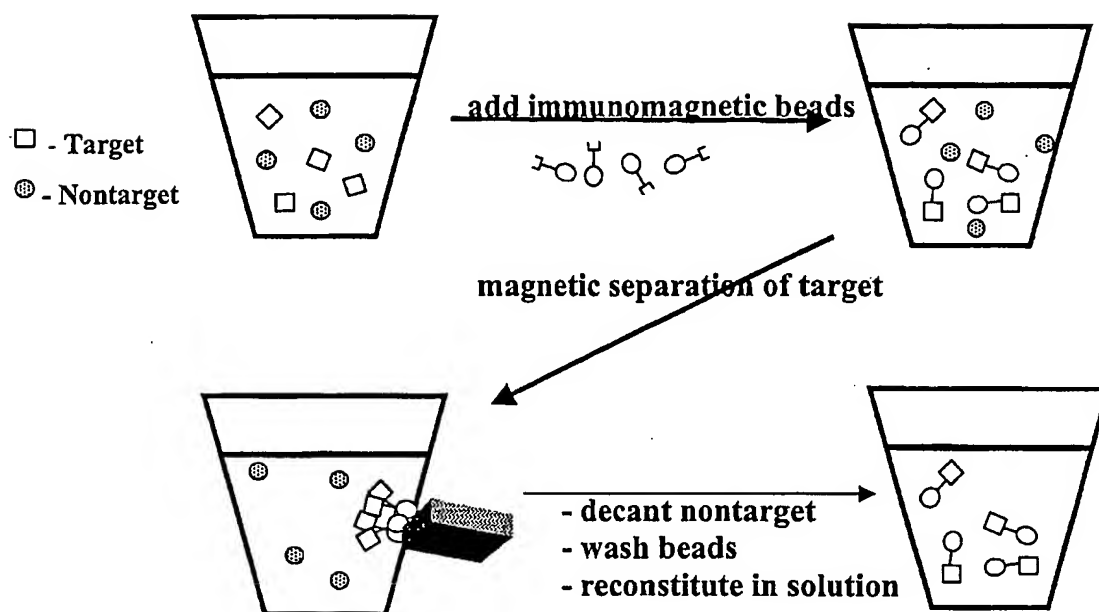


FIGURE 3

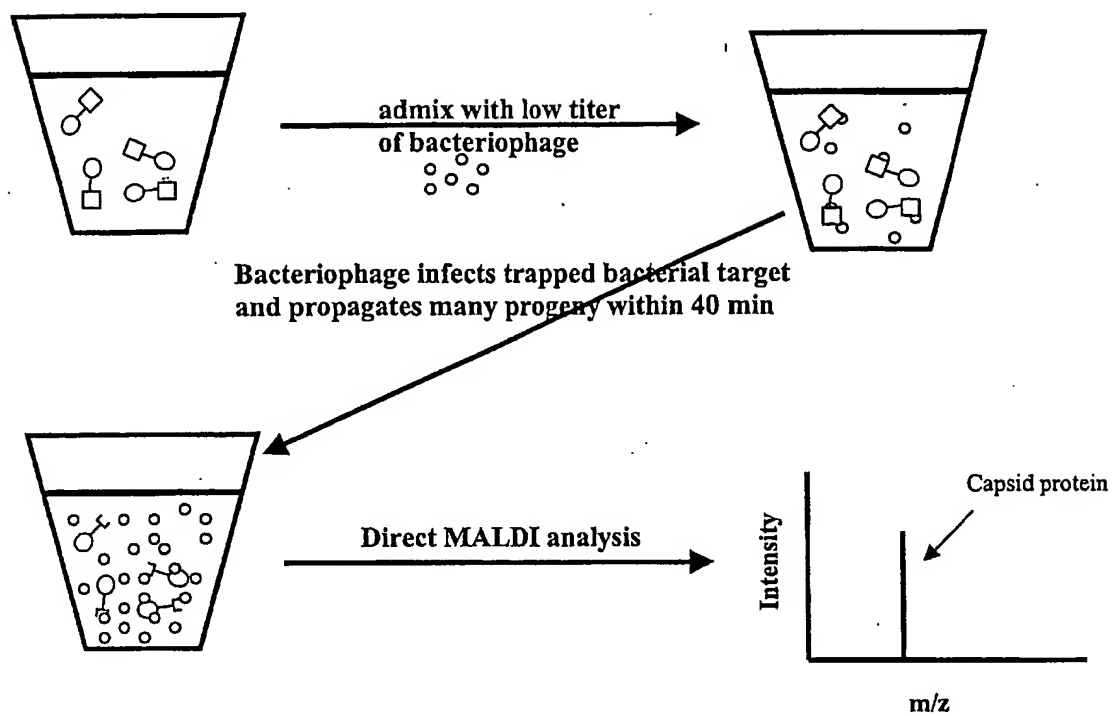


FIGURE 4

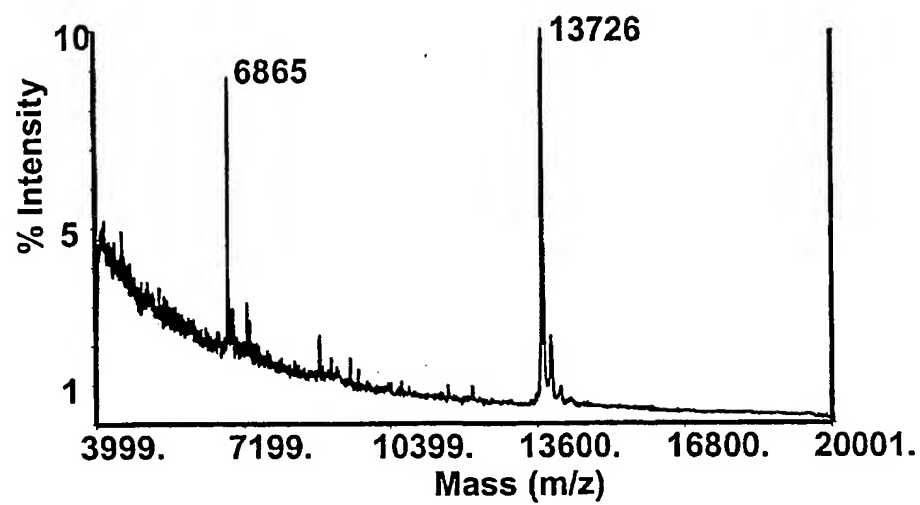


FIGURE 5

